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IN VIVO PRECONDITIONING WITH NORMOBARIC HYPEROXIA INDUCES ISCHEMIC TOLERANCE PARTLY BY TRIGGERING TUMOR NECROSIS FACTOR-α CONVERTING ENZYME/TUMOR NECROSIS FACTOR-α/NUCLEAR FACTOR-κB

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Abstract—Recent studies suggest that intermittent and prolonged normobaric hyperoxia (HO) results in brain ischemic tolerance (BIT), reducing ischemic brain injury. We have attempted to determine the time course of HO-induced BIT, and to explore the putative roles of tumor necrosis factor-α (TNF-α) converting enzyme (TACE), TNF-α, and nuclear factor-κB (NF-κB) activation in mediating this effect. Two core experimental protocols were applied to rats (experiments 1 [E1] and 2 [E2] respectively).

E1 rodents comprised six subgroups, breathing room air (RA; O_2=21%), or 95% oxygen (HO) for 4, 8, 16 h (4RA, 8RA, 16RA and 4HO, 8HO, 16HO respectively). E2 rodents were divided into subgroups, exposed to 95% inspired HO for 4 h/day for six consecutive days (intermittent hyperoxia, InHO) or for 24 continuous hours (prolonged hyperoxia, PrHO). Each of these had a control group exposed to 21% oxygen in the same chamber.

Twenty-four hours after pretreatment, each group was randomly divided to receive 60 min right middle cerebral artery occlusion (MCAO-operated), sham-operation (without MCAO), or no operation (intact). After 24 h reperfusion, neurologic deficit score (NDS), brain water content, Evans Blue extravasation (as a marker of blood–brain barrier permeability), TACE expression, serum TNF-α, and phosphor- $\kappa B\alpha$ levels were assessed in all animals, and infarct volume in the MCAO-operated subgroups.

E1: Compared with the control (RA) group, infarct volume was reduced by 58.6% and 64.4% in 16 h and 24 h respectively. NDS and Evans Blue extravasation was also reduced in 16 h and 24 h. There was no statistical difference among 4 h and 8 h.

E2: Preconditioning with prolonged and intermittent HO decreased NDS, infarct volume and upregulated TACE and increased phosphor-$\kappa B\alpha$ and serum TNF-α level significantly.

Although further studies are needed to clarify the mechanisms of brain ischemic tolerance, InHO and PrHO may partly exert their effects via triggering TACE/TNF-α/NF-κB.

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Abbreviations: AP, activator protein; BBB, blood–brain barrier; BIT, brain ischemic tolerance; DW, dry weight; EB, Evans Blue; HO, hyperoxia; InRA, intermittent room air; IPC, ischemic preconditioning; IT, ischemic tolerance; IκB, inhibitory κB; MCAO, middle cerebral artery occlusion; NDS, neurologic deficit score; NF-κB, nuclear factor-κB; PrRA, prolonged room air; RA, room air; ROS, reactive oxygen species; SOD, superoxide dismutase; TACE, tumor necrosis factor-α converting enzyme; TNF-α, tumor necrosis factor-α; WW, wet weight.

Key words: ischemic preconditioning, ischemic tolerance, normobaric hyperoxia, NF-κB, TNF-a, TACE.

Ischemic tolerance (IT) is an endogenous phenomenon in which brief periods of ischemia render a tissue more resistant to subsequent severe ischemic injury (Romera et al., 2004). This phenomenon (ischemic preconditioning, IPC) has been demonstrated in a variety of organs including brain (Kitagawa et al., 1990). IPC is clearly an attractive target for therapeutic development, and can be induced by means other than simple ischemia, such as exposure to diverse pharmacological agents, changes in inspired oxygen tension, and lipopolysaccharide-induced low-grade inflammation (Valen, 2003). Specifically, hypoxia (Gidday et al., 1994), ischemia (Kitagawa et al., 1990), anoxia (Perez-Pinzon et al., 1996), oxidative stress (Ohtsuki et al., 1992), inhibitors of oxidative phosphorylation (Riepe et al., 1997), and normobaric hyperoxia (HO) (Bigdeli et al., 2007) all induce global or tolerance (brain ischemic tolerance, BIT) to global or local cerebral ischemia.

Recent studies shows that BIT is mediated by the synthesis of proteins which promote neuronal survival, including heat shock protein 70 (Nishi et al., 1993), Bcl-2 (Shimazaki et al., 1994), glutamate transporters (Pradillo et al., 2006), and superoxide dismutase (SOD) (Toyoda et al., 1997).

Nuclear factor-κB (NF-κB) is a dimeric transcription factor that is present in the cytosol in an inactive form bound to its inhibitory protein, inhibitory κB (IκB). When activated by a variety of stimulus, IκB is phosphorylated, ubiquitinated, and generally degraded in the proteosome releasing active NF-κB, this translocates to the nucleus and binds to a consensus site present within the promoter regions of genes such as those for proinflammatory cytokines (Rahman et al., 2001).

NF-κB is activated by various intracellular signals, including cytokines, TNF-α, neurotrophic factors, and neurotransmitters. It is also rapidly activated by HO identified in the lung and heart within 2 and 5 min respectively (Tahepold et al., 2003). Inhibition of NF-κB prior to HO abolishes protection (Leong and Karsan, 2000). NF-κB is a transcription factor for tumor necrosis factor-α (TNF-α) which, in turn, induces activation of NF-κB in a positive feedback loop (Coward et al., 2002). TNF-α is activated on the cell surface by the tumor necrosis factor-α converting enzyme (TACE), thereafter released as a soluble factor, which trimerizes to interact with its two cellular receptors p55 (receptor 1) and p75 (receptor 2) (Lecour et al., 2002).

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Madrigal et al. (2002) have shown TNF-α to be involved in stress-induced expression of iNOS via an NF-κB dependent mechanism, while activation of TACE is dependent on glutamate receptor activation.

Reactive oxygen species (ROS) also play a critical role in activating the signal transduction pathways involving NF-κB, activator protein (AP)-1, mitogen-activated protein kinesis (MAPK) and phosphoinositide-3-kinase (PI-3K), leading to enhanced gene expression of pro-inflammatory mediators (Rahman et al., 2001; Rahman and MacNee, 2000).

Such pathways (involving TACE/TNF-α/TNFR1/NF-κB) are thus implicated in both *in vivo* and *in vitro* models of IPC induced by short ischemia episodes (Pradillo et al., 2006). However, they lack capacity for direct clinical translation. For this reason, safe non-pharmacological stimuli have been sought. One such is normobaric HO, shown to induce neuroprotection against ischemic injury (Bigdeli et al., 2007; Dong et al., 2002). In our laboratory, we have recently shown that pretreatment with intermittent and prolonged HO upregulates glutamate transporters in the rat brain (Bigdeli et al., in press) and confers different degrees of neuroprotection in the rat brain. Intermittent HO also reduces brain edema and Evans Blue (EB) extravasation significantly (Bigdeli et al., 2007).

This study was designed to (i) explore the necessary duration of HO to induce BIT in a focal cerebral ischemic rat model and (ii) to explore the association of such BIT with changes in TACE, serum TNF-α, and NF-κB levels.

EXPERIMENTAL PROCEDURES

Animals and group assignment

All experimental animal procedures were conducted with the approval of the Ethics Committee of the Tarbiat Modares University of Iran. Every effort was made to minimize the number of animals used and their suffering. This study consisted of two experiments.

Experiment 1. One hundred twenty-six male Sprague–Dawley (250–350 g) rats were randomly assigned (21 per group) to one of six subgroups exposed to a chamber containing room air (RA; O_2=21%) or 95% oxygen (HO) for 4, 8 and 16 h (4RA, 8RA, 16RA and 4HO, 8HO, 16HO respectively). Animals were then placed in ordinary RA for a further 24 h, after which all were subjected to 60 min of middle cerebral artery occlusion (MCAO). Twenty-four hours later, neurobehavioural studies were performed before the animals from each group were split into three subgroups of seven, each of which was killed and study made of infarct volume, brain edema, and blood–brain barrier [BBB] permeability respectively.

Three additional groups (each of 14 animals) underwent the RA/HO protocols for 4, 8, and 16 h, but underwent surgery without MCAO. When killed, these sham-operated animal groups were divided into two subgroups (n=7 in each) for evaluation of brain edema, and BBB permeability respectively.

Experiment 2. Eighty-four male Sprague–Dawley (250–380 g) rats were divided randomly into four main groups of 21 animals. Two of these groups were placed in an environmental chamber and exposed to a hyperoxic atmosphere (95% oxygen: normobaric hyperoxic groups, or HO) either intermittently (for four continuous hours of each day for each of six consecutive days, yielding a total hyperoxic exposure of 24 h) or for 24 h continuously. The two other groups were similarly placed in the environmental chamber and exposed to RA equivalent (21% oxygen: normobaric normoxic groups, RA) for similar time periods: the full 6 days ('intermittent' room air, InRA) or for just 24 h ('prolonged' room air, PrRA). Each main group was subdivided into MCAO-operated (monitored by laser Doppler flow meter [MBF3, Moor Instruments, Axminster, UK]), sham-operated, and intact subgroups (n=9, n=6, and n=6 respectively). Animals were then placed in ordinary RA for a further 24 h, after which MCAO-operated subgroups were subjected to 60 min of MCAO. Twenty-four hours later, neurobehavioral studies and then infarct volume measurement were performed. In each sham-operated subgroup (S-InRA, S-PrRA, S-InHO, and S-PrHO), all steps were similar to prolonged or intermittent groups, except of MCAO. In each intact subgroup, all steps were similar to prolonged and intermittent groups without any surgery procedure (I-InRA, I-PrRA, I-InHO, and I-PrHO). Forty-eight hours after pretreatment, animals were killed, neurologic deficit score (NDS) and infarct volume assessed in the MCAO-operated animals, and serum TNF-α, TACE expression, and phosphor-IκBα levels in all others.

In a subset of animals, arterial blood gas analysis was performed just prior to removal from the environmental chamber.

Environmental chamber

All rats underwent adaptation for 1 week in the animal room. The environmental chamber comprised an airtight box (650×350×450-mm) with a gas inlet and outlet port. Internal pressure was continuously monitored by a manometer. Oxygen (90%) or RA (by an aquarium pump) were delivered at a rate of $<$5 l/min through the inlet port. The oxygen concentration inside the container was continuously monitored (Lutron-Do5510 oxygen sensor, Taiwan), and carbon dioxide cleared using soda lime (BDH Limited, Poole, UK) at the bottom of the container. Oxygen concentration was maintained at 90% or 21% according to experimental protocol.

Focal cerebral ischemia

The rats were weighed and anesthetized with 400 mg/kg chloral hydrate (Merck, Germany). MCAO was performed as described by Longa et al., 1989. Briefly, under a surgical microscope, a 3-0 silicone coated nylon suture was introduced through the ECA (external carotid artery) stump. The occluder was advanced into the ICA (internal carotid artery) 20–22 mm beyond the carotid bifurcation until mild resistance indicated that the tip was lodged in the anterior cerebral artery and blocked the blood flow to the MCA (middle cerebral artery). Reperfusion was started by withdrawing the suture after 60 min of ischemia. Rectal temperature was monitored (Citizen-513w) and maintained at 37.0 °C by surface heating and cooling during surgery.

Neurobehavioral evaluation

After the suture was withdrawn, the rats were returned to their separate cages. Twenty-four hours after pretreatment, the rats were assessed neurologically by an observer who was blind to the animal groups. The neurobehavioral scoring was performed using a six-point scale as was previously described by Bederson et al., 1986: normal motor function=0; flexion of contralateral forelimb upon suspended vertically by tail or failure to extend forepaw=1; circling to the contralateral side but have normal posture at rest=2; loss of righting reflex=3; no spontaneous motor activity=4. If the rats died due to subarachnoid hemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study (rate of mortality: 22.3%).

Infarct volume assessment

After kill with chloral hydrate (800 mg/kg), the animals of the MCAO-operated subgroup were decapitated and the brains rap-